The fatty acids in unremodelled trypanosome glycosyl-phosphatidylinositols

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Glycolipid A, the precursor of the glycosyl-phosphatidylinositol (GPI) anchor of the trypanosome variant surface glycoprotein, is constructed in two phases. First, the glycan is assembled on phosphatidylinositol (PI), yielding a glycolipid termed A′. Second, glycolipid A′ undergoes fatty acid remodelling, by deacylation and reacylation, to become the dimyristoyl species glycolipid A. In this paper, we examine the fatty acid content of glycolipid A′ and its cellular progenitors. A′ contains exclusively stearate at the *sn*-1 position and a complex mixture of fatty acids (including 18:0, 18:1, 18:2, 20:4 and 22:6) at *sn*-2. Presumably these fatty acids derive from stearate-containing PI species which initially enter the biosynthetic pathway. We compared the diacylglycerol species from glycolipid A′ with those from phosphatidylinositol to determine whether a subset of stearate-containing PIs is utilized for GPI biosynthesis. We found that the spectrum of stearate-containing diacylglycerols in PI is similar to that in A′, although the proportions of each compound differ. Total PI in general was highly enriched in stearate-containing species. Differences in composition between glycosylated PI and total cellular PI may be due to the substrate specificity of the sugar transferase which initiates the GPI biosynthetic pathway. Alternatively, the species of PI present at the endoplasmic reticulum site of GPI biosynthesis may differ from those in total PI.

INTRODUCTION

Numerous surface proteins and carbohydrates are anchored to the plasma membrane by glycosyl-phosphatidylinositols (GPIs) [reviewed in Cross (1990), Lisanti et al. (1990), Ferguson (1991), Doering (1993) and Englund (1993)]. These structures are found in organisms ranging from yeast to man, and are particularly abundant in lower eukaryotes such as protozoan parasites. The best studied anchor is that of the variant surface glycoprotein (VSG) of *Trypanosoma brucei*, the African trypanosome. The VSG anchor is initially constructed as a free GPI precursor, termed glycolipid A (Krakow et al., 1986) [also called P2 (Menon et al., 1988)], which is subsequently attached to the protein C-terminus (Udenfriend et al., 1991; Caras, 1991). Work with a trypanosome cell-free system has elucidated the pathway of glycolipid A biosynthesis (Masterson et al., 1989, 1990; Menon et al., 1990). (When not otherwise specified, trypanosome refers to the bloodstream form of this parasite, found in its mammalian host.) Phosphatidylinositol (PI) is first converted into glucosaminyl-PI by the addition, and subsequent deacetylation, of *N*-acetylglucosamine (Doering et al., 1989). Three mannose residues and ethanolamine phosphate are then added to the glucosaminyl-PI, completing the core glycan portion of the anchor, and yielding a glycolipid termed A′ (Masterson et al., 1989). Finally, glycolipid A′ is converted into glycolipid A by a highly specific fatty acid remodelling process, in which myristate replaces the fatty acids originally present on the glycosylated PI (Figure 1; Masterson et al., 1990; Doering et al., 1990a). The first step of this remodelling process is deacylation of glycolipid A′ to form the *lyso* compound glycolipid *θ*. This species is reacylated with myristate to form glycolipid A″, which is in turn deacylated and reacylated with a second myristate to complete glycolipid A.

We have been interested in defining the events involved in fatty acid remodelling. As part of these efforts, previous experiments from our laboratory utilized a trypanosome cell-free system to introduce [^{3}H]myristate into glycolipid A″. Glycolipid A″ then contained [^{3}H]myristate at *sn*-2 and an unknown fatty acid, derived from earlier intermediates, at *sn*-1 (Figure 1). The [^{3}H]myristate-labelled diacylglycerol (DAG) of A″ behaved as a unique species with the structure *sn*-1-stearoyl-2-[^{3}H]myristoyl-glycerol, suggesting that its *sn*-1 position was occupied by stearate (Masterson et al., 1990). As glycolipid A″ is formed by addition of myristate to the precursor *θ*, we postulated that the single fatty acid present in glycolipid *θ* was stearate. Extending this logic backward through the pathway, we further speculated that glycolipid A′, as a precursor of glycolipid *θ*, must also contain this fatty acid. In this study we address this hypothesis and show that glycolipids A′ and *θ* do indeed contain stearate. We further investigate glycolipid A′ and examine the fatty acids at its *sn*-2 position. Finally, we compare the fatty acid content of glycolipid A′ with that of its cellular progenitor, PI. This comparison addresses whether a subset of cellular PI species is selected for utilization in GPI biosynthesis.

EXPERIMENTAL

Metabolic radiolabelling of trypanosomes

Cloned ILTat 1.3 trypanosomes were isolated from mouse blood (Bangs et al., 1985) and washed once in radiolabelling medium (Doering et al., 1990b; Hamm et al., 1990). This medium is MEM *α* (Gibco Laboratories), each litre being supplemented with 4.4 g of glucose, 3.9 mg of thymidine, 13.5 mg of hypoxanthine, 68 mg of phenylalanine, 64 mg of tyrosine, 10 g of essentially fatty acid-free BSA (Sigma Chemical Co.), 110 mg of

Abbreviations used: VSG, variant surface glycoprotein; GPI, glycosyl-phosphatidylinositol; PI, phosphatidylinositol; DAG, diacylglycerol; FAME, fatty acid methyl ester; GlcNAc, *N*-acetylglucosamine; GlcN-PI, glucosaminyl-PI; Man, mannose; 14:0, myristate; 16:0, palmitate; 18:0 stearate; PARP, procyclic acidic repetitive protein.

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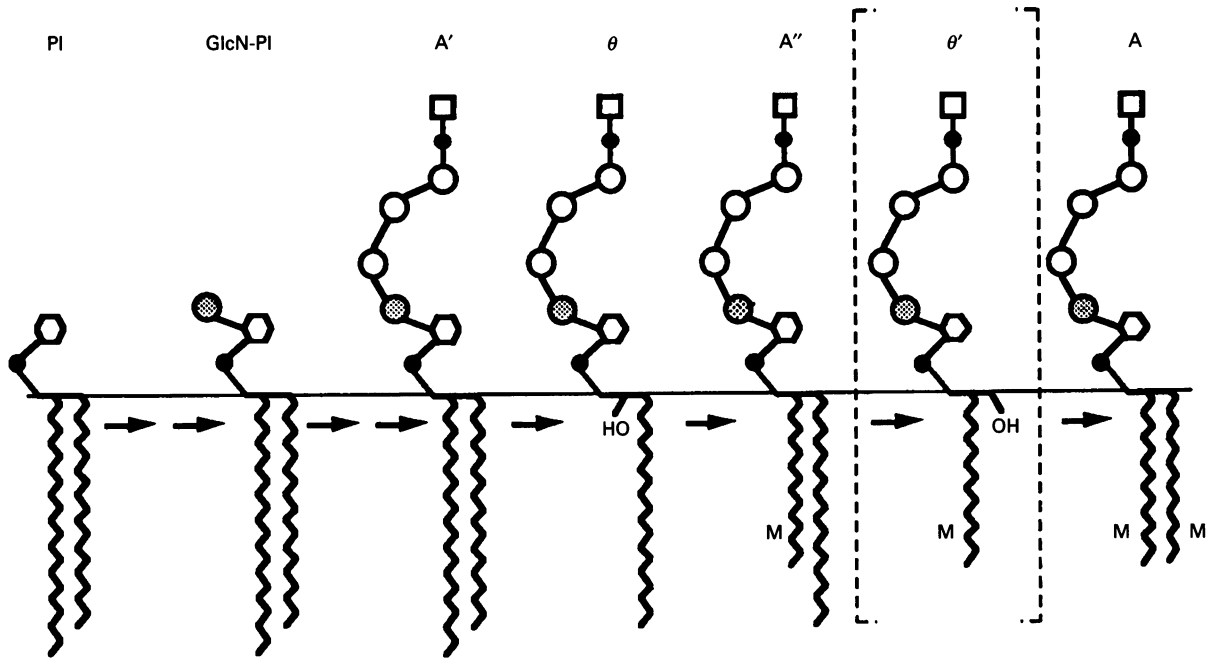

Figure 1 Abbreviated pathway of trypanosome glycolipid A synthesis

●, Phosphate; ⬡, inositol; ◍, glucosamine; ○, mannose; □, ethanolamine; ⦚, fatty acids. A, A′, A″, θ, and θ′, are GPI species described in the text. M indicates myristate, and GlcN-PI is glucosaminyl-PI. As reported in Masterson et al. (1990) and the present paper, the *sn*-1 fatty acid on A′, θ and A″ is exclusively stearate. Glycolipid θ′, in dashed brackets, has been isolated and characterized from trypanosome cell-free reactions (L. U. Buxbaum, unpublished work), but its role in the biosynthetic pathway has not been directly demonstrated.

pyruvate, 50000 units of penicillin, 50 mg of streptomycin and 50 ml of heat-inactivated fetal calf serum (Gibco). Cell viability was assessed by phase microscopy, and cells were incubated for 2–3 h (37 °C; 5% CO_2) in the same medium containing 50–100 μCi/ml of [9,10-^{3}H]stearate (American Radiolabeled Chemicals Inc.; 30 Ci/mmol; dissolved at 10 μCi/μl in ethanol for addition to medium). Trypanosomes radiolabelled with [^{3}H]stearate were hypotonically lysed, and their membranes were washed and resuspended [as in Masterson et al. (1989)] for use in GPI-biosynthesis reactions.

Lipid extraction and t.l.c.

Lipids from cell-free reactions were extracted as in Masterson et al. (1989) and chromatographed on Kieselgel 60 plates (EM Sciences) in solvent A (chloroform/methanol/water, 10:10:3, by vol.). Radiolabelled species were localized by scanning sample and standard tracks of the thin-layer plate with a Bioscan System 200 Imaging Scanner. To recover glycolipids θ and A′, silica was scraped from appropriate areas of the plate, and lipids were extracted sequentially in solvent B (chloroform/methanol, 2:1, v/v), solvent C (chloroform/methanol/water; 55:45:10, by vol.), and two to three times with solvent A. Radiolabelled PI was recovered by three extractions of silica with solvent B, and radiolabelled DAG by three extractions with chloroform/methanol (9:1, v/v).

RESULTS

What are the fatty acids on trypanosome GPIs at the start of remodelling?

Our initial objective was to provide direct evidence for the presence of stearate in glycolipids A′ and θ, a possibility suggested by our earlier experiments (Masterson et al., 1990). To radiolabel these species with [^{3}H]stearate, we exploited the fact that GPI intermediates readily accumulate in the cell-free system for GPI biosynthesis (Masterson et al., 1989), even though they are present at extremely low concentrations in living trypanosomes. We incubated trypanosomes in medium containing [^{3}H]stearate to radiolabel cellular PI, a substrate for the first reaction in GPI synthesis (see Figure 1). Under the conditions used, less than 2% of the [^{3}H]stearate is elongated to other species and none is oxidized or desaturated (results not shown). We then lysed the cells hypotonically, washed the membranes, and incubated them with the nucleotide sugars required for GPI biosynthesis *in vitro*. We did not add the myristoyl-CoA which is needed for fatty acid remodelling. Under these conditions, GPI biosynthesis can only proceed up to the formation of glycolipid θ, the first *lyso* species (Figure 1). As shown in Figure 2 (lane 2), a number of lipids were metabolically labelled during incubation of the trypanosomes with [^{3}H]stearate. Glycolipids A and C were also radiolabelled because the commercial [^{3}H]stearate used in this experiment contained traces of contaminating [^{3}H]myristate [< 2% by fatty acid methyl ester (FAME) analysis; results not shown], which is preferentially incorporated into these GPI species. [Glycolipid C is a GPI intermediate identical with glycolipid A except that it contains an extra fatty acid linked to inositol (Krakow et al., 1989; Mayor et al., 1990)]. As shown below, myristate was not incorporated into A′ and θ. No alteration of this lipid profile was observed when only one of the nucleotide sugars needed for GPI biosynthesis was included in the reaction (Figure 2, lanes 3 and 4). However, when both UDP-GlcNAc and GDP-Man were added to the washed trypanosome membranes (Figure 2, lane 5), two additional [^{3}H]stearate-labelled species were formed. As expected, these species co-migrated with [^{3}H]mannose-labelled glycolipids A′ and θ (lane 1). They also were degraded by

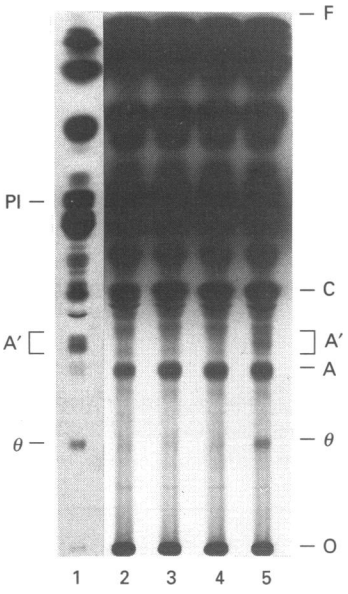

Figure 2 Incorporation of stearate into GPI-biosynthetic intermediates A′ and θ

Membranes from trypanosomes labelled *in vivo* with [^{3}H]stearate (5×10^7 cell equivalents in a 125 μl reaction) were incubated at 37 °C for 0 (lane 2 only) or 15 min with the following additions: lane 1, 0.5 mM UDP-GlcNAc and 1 μCi GDP-[^{3}H]Man (New England Nuclear; 30 Ci/mmol); lane 2, no additions; lane 3, 0.5 mM UDP-GlcNAc; lane 4, 0.5 mM GDP-Man; lane 5, 0.5 mM UDP-GlcNAc and 0.5 mM GDP-Man. Lipids were extracted and chromatographed on Kieselgel 60 plates in solvent A as in Masterson et al. (1989). GPI species are indicated. O, origin; F, front. Glycolipids A and C (Krakow et al., 1989; Mayor et al., 1990) are radiolabelled because [^{3}H]myristate, a contaminant in the [^{3}H]stearate, was specifically incorporated into these GPIs. This is not the source of radiolabel present in A′ and θ, as shown in later experiments. Lane 1 is from a 13 h exposure of this t.l.c.; other lanes are from a 63 h exposure. The apparent overlap of PI with other species is due to the long exposure time of this fluorograph. Shorter exposures (not shown) indicate that it is separate from other radiolabelled species, allowing its isolation in radiochemically pure form from similar t.l.c. plates for experiments presented later in this paper.

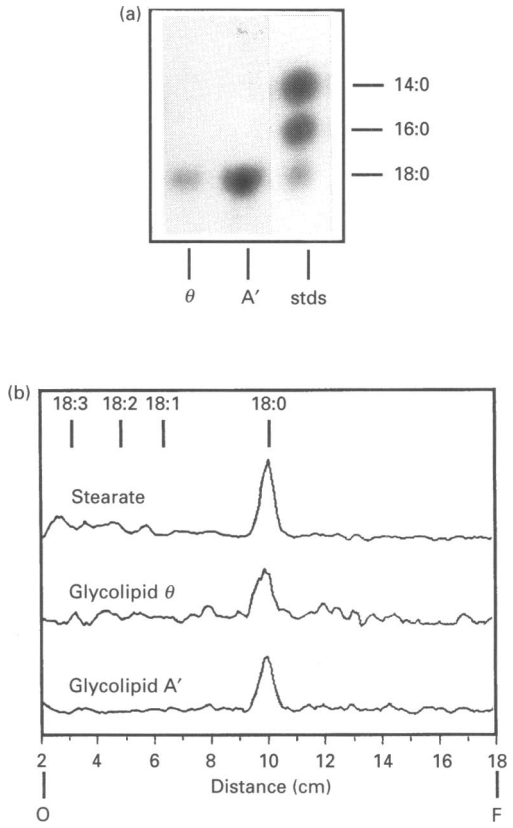

Figure 3 FAME analysis of [^{3}H]stearate-labelled GPI-biosynthetic intermediates

Glycolipids A′ and θ were recovered (see the Experimental section) from t.l.c. plates like that in Figure 2, lane 5, and portions were rechromatographed to assess purity. Radiochemically pure GPIs or radiolabelled fatty acid standards were dried, treated with 2% H_2SO_4 in methanol (1.5 h; 70 °C), and extracted twice with pentane. Pooled pentane phases were washed with distilled water, dried and resuspended in 20 μl of chloroform/methanol (9:1) for t.l.c. analysis. (**a**) To assess chain length, the FAMEs were analysed on reversed-phase plates (Analtech, Inc., Newark, DE, U.S.A.) developed in chloroform/methanol/water (5:15:1, by vol.). Part of a fluorograph of a t.l.c. plate is shown, with the positions of standards indicated. No radiolabelled species were detected elsewhere on the plate. A′ and θ indicate FAMEs prepared from those GPIs; stds are FAMEs prepared from the indicated species. (**b**) To resolve species by degree of unsaturation, FAMEs were analysed on 20% argentation t.l.c. plates (Analtech), developed in hexane/ether/acetic acid (95:5:1, by vol.) at 4 °C for 1 h. The positions of standards are indicated. Radioactivity was detected using a Bioscan System 200 Imaging Scanner (Bioscan Corp.). Data were collected for 1 h per track, and 256 point spectra smoothed with a three-point smoothing routine are shown.

treatment with PI-specific phospholipase C and GPI-specific phospholipase D, confirming that they were GPI species (results not shown). To determine whether the ^{3}H-labelled fatty acids in these species were still stearate or had been metabolically altered, we generated ^{3}H-labelled FAMEs from glycolipids A′ and θ and analysed them by reversed-phase (Figure 3a) and argentation (Figure 3b) chromatography. The radiolabel in these species was all in the form of [^{3}H]stearate, with no apparent desaturation or change in chain length. The possible presence of unsaturated species in the argentation chromatography (Figure 3b) is ruled out by the reversed-phase chromatography (Figure 3a) in which they would have a higher R_F value. Results comparable with those with A′ and θ were also obtained with PI (results not shown).

When glycolipid θ is radiolabelled in the glycan portion with [^{3}H]mannose, it migrates as a discrete band in several solvent systems, including solvent A (Figure 2, lanes 1 and 5) and a basic solvent system (chloroform/methanol/1 M ammonium acetate/15 M NH_4OH/water, 180:140:8:9:23, by vol.) (results not shown). It is also well resolved (6% difference in R_F value in solvent A) from the *lyso*-GPI intermediate θ′ (see Figure 1) which contains myristate as its sole fatty acid (Masterson et al., 1990, L. U. Buxbaum, personal communication). Because of this behaviour, glycolipid θ appears to be a homogeneous *lyso* species which contains exclusively stearate. Glycolipid θ is derived from glycolipid A′, and co-migrates on t.l.c. with the unique phospholipase A_2 product of that GPI (Masterson et al., 1990). Therefore the *sn*-1 position in glycolipid A′ must also be occupied by stearate.

Unlike glycolipid θ, glycolipid A′ appears heterogeneous by t.l.c. (Figure 2, lanes 1 and 5), suggesting that the *sn*-2 position of that glycolipid is occupied by a mixture of fatty acids. This is an important observation, as it provides a clue to the structure of early GPI-biosynthetic intermediates. We therefore wished to characterize these fatty acids. Because it is not possible to purify mass amounts of GPI-biosynthetic intermediates, we used an approach exploiting [^{3}H]stearate radiolabelling which was designed to avoid potential contamination with other lipid species. We eluted [^{3}H]stearate-labelled A′ from a t.l.c. plate like that in Figure 2 (lane 5) and released the DAG moiety by

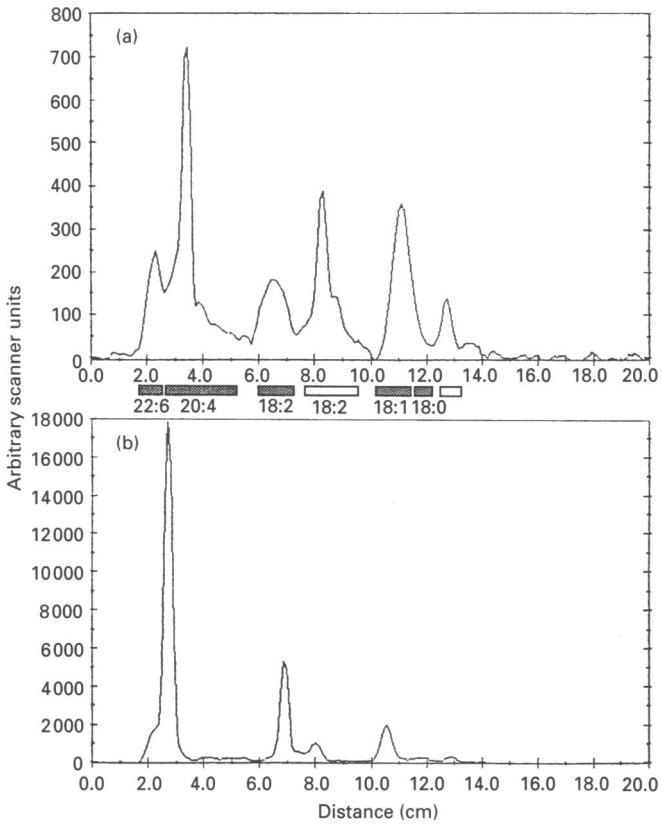

Figure 4 Analysis of [^{3}H]stearate-labelled DAG from glycolipid A′ (a) and cellular PI (b)

Cellular PI and glycolipid A′ were purified as described in the Experimental section. For analysis, each was digested as in Masterson et al. (1989) with PI-specific phospholipase C from *Bacillus thuringiensis* (a generous gift from Martin Low, Columbia University, New York, NY, U.S.A.). The digest was then chromatographed as in Figure 2, and the released [^{3}H]stearate-labelled DAGs were recovered from near the solvent front of plates like that shown in Figure 2. DAGs of glycolipid A′ or PI were applied to 5% argentation plates (Analtech) which were developed in chloroform/methanol (99:1, v/v) (Masterson et al., 1990). The plate was chromatographed for 35 min, removed and allowed to dry, and then rechromatographed for 35 min in the same tank. Non-radioactive standards chromatographed on the same plate were detected by spraying with 0.12% rhodamine in distilled water and examination under u.v. light. Shaded bars represent the positions of migration of standard DAGs, with the number beneath each bar indicating the fatty acid paired with stearate. The open bars represent 1,3 forms of standard DAGs. The species at 12.7 cm in (**a**) was not directly identified in this experiment, but other experiments suggest that it is the 1,3 form of 18:0–18:1. Scans of t.l.c. plates (10 h per track; data smoothed as in Figure 3b) are shown, with the ordinate marked in arbitrary scanner units.

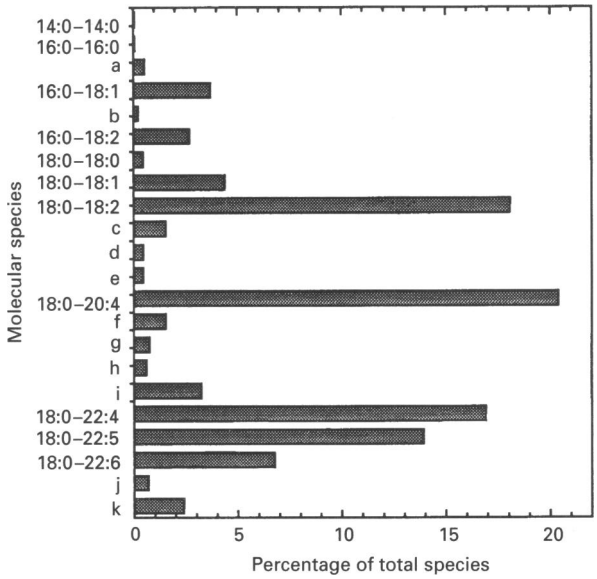

Figure 5 Molecular species of DAG from trypanosome PI

Using methods described in Pessin and Raben (1989), trypanosome phospholipids were fractionated by t.l.c., and cellular PI was further purified by h.p.l.c. Briefly, dried organic extracts of trypanosomes were dissolved in chloroform/methanol (9:1, v/v), and applied to a 500 μm silica-gel G t.l.c. plate. The plate was developed in isopropyl ether/acetic acid (96:4, v/v), and phospholipids were located by comparison with co-chromatographed standards. The appropriate region of the plate was scraped off, the recovered silica was extracted with chloroform/methanol (9:1, v/v) and the extract was chromatographed on a Zorbax PRO-10 Sil h.p.l.c. column (Dethloff et al., 1986). Individual phospholipids were hydrolysed with phospholipase C from *Bacillus cereus*, and released DAGs were isolated and derivatized with *t*-butyldimethylchlorosilane. The resulting *t*-butyldimethylsilyl DAG esters were recovered into hexane and analysed by gas chromatography on a fused silica capillary column (15 m × 0.32 mm) coated with SP-2380 liquid phase (Pessin and Raben, 1989). The abscissa indicates the percentage of total DAGs present as each molecular species, with species shown at the left in order of elution (di-14:0 is eluted earliest). Identification of most major species was accomplished by relative retention time and comparison with standards; unidentified species are indicated by letters.

treatment with PI-specific phospholipase C. The specificity of this enzyme precludes cleavage of any contaminating species. The DAG products of the lipase digestion (which generally went to at least 80% completion) were extracted and purified by t.l.c. in solvent system A, to separate them from uncleaved compounds and any other radiolabelled lipids that might have contaminated the starting material. DAGs were then recovered from the thin-layer plates and analysed by argentation t.l.c. (Figure 4a). This analysis showed the presence of DAGs containing stearate paired with either the shorter fatty acids 18:0, 18:1 and 18:2 (56% of the total) or longer unsaturated fatty acids such as 20:4 and 22:6 (44% of the total). This t.l.c. method provides limited resolution of these latter DAGs, but we could not obtain sufficient radiolabelled material for more highly resolving techniques. As shown, there was some conversion of 1,2- into 1,3-DAG during sample preparation, confirmed by reversed-phase chromatography with appropriate standards [t.l.c. performed as in Masterson et al. (1990); results not shown. Although reversed-phase chromatography resolves 1,2 and 1,3 species very clearly, the resolution of individual 1,2 species from each other is poor].

Comparison of fatty acids from glycolipid A′ with those of PI

The fatty acids on A′ presumably derive from the PI which was the initial substrate for GPI biosynthesis. We wanted to determine if these PI species were chosen randomly, or whether a particular subset was directed to GPI biosynthesis. To accomplish this we compared the DAGs of A′ with those of total PI from trypanosomes. To simplify this analysis, we first examined PI radiolabelled *in vivo* with [^{3}H]stearate, to permit a direct comparison of stearate-containing species. As shown in Figure 4, the major species are common to both A′ and PI. However, there appears to be a trend in A′ towards shorter and more highly saturated compounds. For example, there is relatively more 18:0–18:2 and 18:0–18:1 in glycolipid A′ (56% compared with 33% in PI), with less material in larger species near the origin of the plate. There is also trace di-18:0 in A′, at the leading edge of the peak at 11 cm (this resolved more clearly in other analyses), although di-18:0 is not detectable in PI. Nonetheless, species containing

longer and more highly unsaturated fatty acids are certainly also utilized in GPI biosynthesis. The fact that the peaks are broader in the DAGs derived from A′ could be due to more heterogeneity in the fatty acids (especially in the highly unsaturated species), to overloading of the t.l.c. plate (necessary because of the low radioactivity available), or to more isomerization to 1,3-forms.

We also compared the A′ fatty acids with those of the entire PI pool, including those species not containing stearate. To do this we isolated total PI from non-radiolabelled cells, digested it with phospholipase C, and analysed the released DAGs by gas chromatography (Figure 5). Trypanosome PI contains predominantly DAG, with alkenyl–acyl only 0.3%, and alkyl–acyl 0.1%, of the total. {In contrast, alkenyl–acyl and alkyl–acyl DAG species comprise at least 5% of other phospholipids and free DAGs [Patnaik et al. (1993) and results not shown].} Overall, stearate-containing species comprise about 80% of the DAGs in PI. Some of the stearate is present in several prominent species typical of phospholipids, such as 18:0–18:1, 18:0–18:2 and 18:0–20:4 (Figure 5). Additionally, a trace amount of di-18:0 was detected, and significant quantities of 18:0–22:4, 18:0–22:5 and 18:0–22:6.

The DAGs of radiolabelled PI more closely resemble the total cellular PI than do the DAGs of glycolipid A′. For example, 18:0–18:1 and 18:0–18:0 constitute 10% of radiolabelled PI (Figure 4b) and 8% of stearate-containing cellular PI (Figure 5), compared with over 23% of DAGs in glycolipid A′ (Figure 4a). Similarly, 18:0–20:4 and the longer and more highly unsaturated fatty acids together comprise 67% of stearate-labelled PI and 63% of total stearate-containing PI species, but only 44% of DAGs in glycolipid A′.

DISCUSSION

The biosynthesis of trypanosome GPIs begins with glycan construction on PI to form glycolipid A′, and concludes with fatty acid remodelling to introduce myristate, forming glycolipid A. In this paper, we have demonstrated that biosynthetic intermediates glycolipids A′ and θ contain exclusively stearic acid. Then, by characterizing the DAGs in [^{3}H]stearate-labelled A′, we have shown that the *sn*-2 fatty acids on A′ are heterogeneous, including 18:0, 18:1, 18:2, 20:4 and 22:6. This fatty acid heterogeneity explains the characteristic smearing of [^{3}H]mannose- or [^{3}H]stearate-labelled glycolipid A′ on t.l.c. plates [Figure 2 and Masterson et al. (1989, 1990)]. In contrast, glycolipids θ, A″ and A, each with a unique configuration of fatty acids, form sharp bands on t.l.c.

Our analysis of GPI intermediates provides a more complete characterization of the fatty acid remodelling process. It also enabled us to address the question of how the fatty acid composition of A′ compares with that of PI, important for understanding the early steps in GPI biosynthesis. Our first approach to this question was to isolate [^{3}H]stearate-labelled PI and to analyse its DAGs by t.l.c. Many [^{3}H]stearate-labelled DAG species are common to A′ and PI. However, A′ seems relatively enriched in shorter and more saturated compounds. We next compared the fatty acids of glycolipid A′ with those of the total cellular PI (including those that were not labelled with [^{3}H]stearate). We find that a large proportion of the PI DAGs do contain stearate, in agreement with observations by Patnaik et al. (1993). Interestingly, large and highly unsaturated species (those eluting later than 18:0–20:4 in our gas-chromatography system) comprise over 40% of PI DAGs, and they are also a significant fraction of free DAGs or DAGs in other trypanosome phospholipids [Patnaik et al. (1993); T. L. Doering, M. S. Pessin, G. W. Hart, D. M. Raben and P. T. Englund, unpublished work). [Species with retention times greater than that of 18:0–20:4 were 47% of DAGs from PI, 50% in phosphatidylcholine, 25% in phosphatidylethanolamine, 14% in phosphatidylserine and 33% in free DAGs (Figure 5 and results not shown).] Such fatty acids constitute less than 1% of the total in the rat serum environment of the trypanosomes (Dixon, 1967; Doering et al., 1993). The large quantities in trypanosomes are either produced by the parasites, possibly by a combination of elongation and desaturation of serum fatty acids, or are selectively imported.

The differences between the fatty acid content of [^{3}H]stearate-labelled PI and glycolipid A′ could be explained in several ways, which are not mutually exclusive. First, the sugar transferase which initiates the pathway could preferentially glycosylate certain PIs. The fact that the 20% of the trypanosome PI species that do not contain stearate are excluded from GPI biosynthesis suggests that this enzyme manifests some degree of selectivity. Perhaps DAGs containing shorter fatty acids paired with stearate are preferred. Alternatively, various cellular compartments could exhibit different PI compositions (White, 1973; Yeagle, 1991), with only those PI species present at the endoplasmic reticulum site of GPI biosynthesis readily available for the sugar transferase. Finally, we cannot rule out the possibility that the fatty acid differences between PI and A′ could be due to remodelling reactions not involving myristate, which occur on intermediates between GlcN-PI and glycolipid A′. The possibility of additional remodelling could be addressed by comparing the fatty acid content of glycolipid A′ with that of GlcN-PI, the first GPI intermediate (Figure 1). Although we were able to radiolabel GlcN-PI with [^{3}H]stearate in reactions similar to those shown in Figure 2, we could not recover sufficient pure material for analysis. Studies of another GPI-anchored protein, the acetylcholinesterase of *Torpedo marmorata*, show that its fatty acid content differs significantly from that of total cellular PI (Butikofer et al., 1990). The reasons for these differences could be the same as those we have considered here.

What is the significance of stearate as the exclusive *sn*-1 fatty acid in A′ and other GPI-biosynthetic intermediates? One possibility is that stearate is needed at some stage of the biosynthetic pathway. This requirement would be met if the sugar transferase that initiates GPI biosynthesis were specific, and did not utilize the small portion of PI species that do not contain stearate. Another possibility is that stearate is important for some other aspect of trypanosome biology. Stearate obviously plays no role in the mature GPI anchor of VSG, as in that anchor it is replaced by myristate. However, it may be important in procyclic trypanosomes, a form present in the tsetse fly vector. Procyclic trypanosomes have no VSG coat, although they do have an abundant GPI-anchored surface protein termed procyclin (Roditi et al., 1989) or procyclic acidic repetitive protein (PARP) (Clayton and Mowatt, 1989). The PARP anchor, which is constructed in the same way as the VSG anchor (Field et al., 1991a), contains an *sn*-1-stearoyl-2-*lyso*-glycerol, exactly like glycolipid θ (Figure 1) (Field et al., 1991b). Stearate is the exclusive fatty acid found on the glycerol moiety of PARP, and may be important for its function. The requirement for stearate in early GPI-biosynthetic intermediates would allow conservation of GPI-biosynthetic machinery between different life-cycle stages of trypanosomes, while still ensuring that PARP received stearate. In both bloodstream and procyclic trypanosomes, processing of the DAG moiety of the GPI may thus proceed identically through the first deacylation step in remodelling, but would continue with myristoylation only in bloodstream forms.

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REFERENCES

Bangs, J. D., Hereld, D., Krakow, J. L., Hart, G. W. and Englund, P. T. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 3207–3211
Butikofer, P., Kuypers, F. A., Shackleton, C., Brodbeck, U. and Stieger, S. (1990) J. Biol. Chem. **265**, 18983–18987
Caras, I. W. (1991) Cell Biol. Int. Rep. **15**, 815–826
Clayton, C. E. and Mowatt, M. R. (1989) J. Biol. Chem. **264**, 15088–15093
Cross, G. A. M. (1990) Annu. Rev. Cell Biol. **6**, 1–39
Dethloff, L. A., Gilmore, L. B. and Hook, G. E. (1986) J. Chromatogr. **382**, 79–87
Dixon, H. (1967) Trans. R. Soc. Trop. Med. Hyg. **61**, 12–13
Doering T. L. (1993) in Cell Surface and Extracellular Glycoconjugates; Structure and Function (Roberts, D. and Mecham, R. P., eds.), pp. 83–117, Academic Press, San Diego
Doering, T. L., Masterson, W. J., Englund, P. T. and Hart, G. W. (1989) J. Biol. Chem. **264**, 11168–11173
Doering, T. L., Masterson, W. J., Hart, G. W. and Englund, P. T. (1990a) J. Biol. Chem. **265**, 611–614
Doering, T. L., Raper, J., Buxbaum, L. U., Hart, G. W. and Englund, P. T. (1990b) Methods **1**, 288–296
Doering, T. L., Pessin, M. S., Hoff, E. F., Hart, G. W., Raben, D. M. and Englund, P. T. (1993) J. Biol. Chem. **268**, 9215–9222
Englund, P. T. (1993) Annu. Rev. Biochem. **62**, 121–138
Ferguson, M. A. J. (1991) Curr. Opin. Struct. Biol. **1**, 522–529
Field, M. C., Menon, A. K. and Cross, G. A. M. (1991a) J. Biol. Chem. **266**, 8392–8400
Field, M. C., Menon, A. K. and Cross, G. A. M. (1991b) EMBO J. **10**, 2731–2739
Hamm, B., Schindler, A., Mecke, D. and Duszenko, M. (1990) Mol. Biochem. Parasitol. **40**, 13–22
Krakow, J. L., Hereld, D., Bangs, J. D., Hart, G. W. and Englund, P. T. (1986) J. Biol. Chem. **261**, 12147–12153
Krakow, J. L., Doering, T. L., Masterson, W. J., Hart, G. W. and Englund, P. T. (1989) Mol. Biochem. Parasitol. **36**, 263–270
Lisanti, M. P., Rodriguez-Boulan, E. and Saltiel, A. R. (1990) J. Membr. Biol. **117**, 1–10
Masterson, W. J., Doering, T. L., Hart, G. W. and Englund, P. T. (1989) Cell **56**, 793–800
Masterson, W. J., Raper, J., Doering, T. L., Hart, G. W. and Englund, P. T. (1990) Cell **62**, 73–80
Mayor, S., Menon, A. K. and Cross, G. A. M. (1990) J. Biol. Chem. **265**, 6174–6181
Menon, A. K., Mayor, S., Ferguson, M. A. J., Duszenko, M. and Cross, G. A. M. (1988) J. Biol. Chem. **263**, 1970–1977
Menon, A. K., Schwarz, R. T., Mayor, S. and Cross, G. A. M. (1990) J. Biol. Chem. **265**, 9033–9042
Patnaik, P. K., Field, M. C., Menon, A. K., Cross, G. A. M., Yee, M. C. and Butikofer, P. (1993) Mol. Biochem. Parasitol. **58**, 97–105
Pessin, M. S. and Raben, D. M. (1989) J. Biol. Chem. **264**, 8729–8738
Roditi, I., Schwarz, H., Pearson, T. W., Beecroft, R. P., Liu, M. K., Richardson, J. P., Buhring, H. J., Pleiss, J., Bulow, R., Williams, R. O. and Overath, P. (1989) J. Cell Biol. **108**, 737–746
Udenfriend, S., Micanovic, R. and Kodukula, K. (1991) Cell Biol. Int. Rep. **15**, 739–759
White, D. A. (1973) in Form and Function of Phospholipids, Volume 3 (Ansell, G. B., Hawthorne, J. N. and Dawson, R. M. C., eds.), pp. 441–482, Elsevier, Amsterdam
Yeagle, P. (1991) The Structure of Biological Membranes, CRC Press, Boca Raton, FL

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